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Endothelial cells from different vascular beds, including tumor vasculature, have been shown to have different behavior and different gene expression. In tumors, abundant fibrin in the extracellular matrix dictates that fibrinolytic capability is required for new vessel formation. We have developed an in vitro breast cancer angiogenesis assay using mammary vessels from mice in a 3-dimensional fibrin matrix. Confocal microscopy of these assays is used to quantitate the number of endothelial cells invading the matrix as well as the length of tubular structures formed by the cells. This assay can be used to investigate fibrinolytic molecules important in vascularization of breast tumors.

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Introduction

We started our studies because of the data that tumors are rich in fibrin and because of the link of urokinase plasminogen activator (uPA) expression to poor prognosis. The latter link has recently been reinforced by new findings[1]. Because there was little data from tumor models or from in vitro assays in fibrin, we felt that our proposed investigations would shed light on the role of the fibrinolytic system in breast cancer angiogenesis. Studies in "knock-out" mice have shown very little change in the vascular system for mice deficient in tissue plasminogen activator (tPa), uPA, urokinase plasminogen activator receptor (uPAR), or plasminogen activator inhibitor 1 (PAI-1)[2]. However, PAI-1 deficient mice were shown to vascularize tumors less efficiently, with a resultant decrease in tumor size when compared to similar tumors in wild-type mice[3]. Additionally, MT1-MMP deficient mice do have a vascular phenotype [4], and this enzyme has been shown to be an important fibrinolytic enzyme. In summary, although the literature implicates the fibrinolytic system as an indicator of poor prognosis, only MT1-MMP deficiency has been shown to produce a vascular phenotype, and only PAI-1 has been implicated as a factor in tumor-associated angiogenesis.

The literature also robustly supports the notion that endothelial cells from vessels in different organs and different types of vessels have specific patterns of gene expression[5]. We therefore felt that aortic ring assays previously done as *in vitro* models of angiogenesis may have been producing data that possibly did not apply to breast cancer endothelium since endothelial cells in these assays do not come from a venule in the breast. Our previous studies showed that breast tumor-associated endothelial cells have strongly upregulated messenger RNA (mRNA) for tPA and MT1-MMP, with some upregulation of uPA expression. Blocking antibodies are available to these proteins, and part of our proposal dealt with adapting the aortic ring assay as an *in vitro* angiogenesis assay for breast cancer using a mammary fat pad vessel as a source of endothelial cells. We have successfully developed the mammary vessel assay and we are currently gathering data using the assay.

The second part of our proposal dealt with applying the knowledge gained from the mammary vessel assays to breast tumors produced in nude mice. This part of the project has not been accomplished since we have not yet gotten the data we need from the mammary vessel assay. We have applied for a no-cost extension of the grant in order to allow time for additional experiments and data analysis of the mammary vessel assays before going to the in vivo tumor production. The body of this report outlines our results to date and indicates how we will fulfill the aims of the project during the no-cost extension.

Body

1. Fluorescence studies and confocal analysis of mammary vessel assays.

As indicated in last year's report, we have been able to identify endothelial tubular structures in the 3-dimensional fibrin matrix by using uptake of DiI-labeled acetylated low-density lipoproteins (DiI-acLDL). Acetylated LDL does not internalize as a result of binding to the LDL receptor but rather because of a scavenger receptor that is specific to endothelial cells[6]. In last year's report, we had been successful in getting this technique to work in mammary vessel explants in a 3-dimensional fibrin matrix. In the past year, we have been working to compare results in this system using mammary vessel explants or aortic ring explants. These studies will confirm our belief that mammary vessels may respond to angiogenic factors differently than aortic rings and validate the specificity of our assay.

1A. Responses of mammary vessels and aortic rings growing in fibrin matrices to angiogenic factors.

As outlined in the original proposal, we first studied the mammary vessel explants in endothelial cell media without additions or with various additives. Table 1 outlines the media used for these experiments. Each assay consisted of 4 chambers in a chambered coverslip. Mammary vessels were placed in fibrin matrices in assays 1-8 and aortic rings were similarly treated in assays 9-16. All 4 chambers were treated with Di-AcLDL for 3 days prior to harvest. Assays were fixed with 4% paraformaldehyde/1.5% Triton-X 100 overnight at 4°C. After washing with PBS, Dent's fixative was applied overnight at 4°C, followed by Dent's fixative plus 6% hydrogen peroxide for 2 hours at room temperature with rocking. Chambers were then washed with 100% methanol twice and incubated at -80° overnight. After two washes with 50% methanol in PBS for 30 minutes at room temperature two similar washes with phosphate-buffered saline (PBS) plus 0.5% Triton-X 100, chambers were stained with Yo-Pro-1 (1:750 in PBS), which binds to nucleic acids and fluoresces green. Depending on the intensity of excitation, Yo-Pro-1 produces intense green nuclear staining and less intense cytoplasmic staining. Fluorescence microscopy was performed with the excitation laser for Yo-Pro-1 set to give nuclear, but not cytoplasmic staining.

Assay number	Growth Factor/conc.
1 & 9	No additives
2 & 10	BPE
3 & 11	VEGF, 50 ng/ml
4 & 12	FGF ng/ml
5 & 13	BPE + VEGF
6 & 14	BPE + FGF
7 & 15	VEGF + FGF
8 & 16	All

Table 1. Additives to fibrin matrices for mammary vessels or aortic rings. Chambers 1-8 were mammary vessels and chambers 9-16 were aortic rings. Basal media was 199E with 10% fetal bovine serum, 0.5 ng/ml EGF, and 3 μM hydrocortisone. BPE, bovine pituitary extract; VEGF, vascular endothelial growth factor; FGF, basic fibroblast growth factor (FGF-2).

Figures 1A and 1B show representative low-power fields (4X objective with 2.5X zoom) of mammary vessel (top row) and aortic ring (bottom row) explants growing in fibrin matrices with treatments as given in Table 1. The explant is the large red structure at the side, top or bottom of each picture. A square with "no sprouts" signifies that none of the four chambers of the chambered cover slip containing the vessel (in all cases,

aortic ring) fragments exhibited sprouting from the explant. Interestingly, the mammary vessels exhibited endothelial sprouting even in media supplemented only with EGF, hydrocortisone, and 10% fetal calf serum while the aortic rings did not. Although supplementation with bovine pituitary extract (BPE) enabled sprouting in both aortic rings and mammary vessels, addition of BPE to VEGF and basic FGF (FGF) supplementation seemed inhibitory in both vessel types. Although representative fields are shown, the differences produced by different treatments and the difference between mammary vessels and aortic rings are more pronounced than can be seen in Figure 1A and 1B. For instance, in Figure 1B with the combination of FGF and VEGF (next to the last column of pictures), the field depicting aortic ring sprouts (bottom row) represents the only sprouting we saw in aortic rings with this treatment. However, the mammary vessels treated with the combination of FGF and VEGF exhibited sprouting in every chamber and at multiple locations from the same explant. We are in the process of devising an image analysis macro using IMAGEJ software (a free download from the NIH) to quantitate the number of sprouting cells. This should reveal quantitative differences between treatments. Additionally, using confocal microscopy, we have acquired high power (40X water-immersion objective) z series on each a number of fields from each chamber. These have been used to generate 3-dimensional projections that show tubular structures formed by the sprouting endothelial cells (Figure 2). These structures can be analyzed as to length and branch points by the ImageJ software. Over the few months, we will be analyzing the structures in the sprouts to develop a more quantitative picture of high the aortic rings and mammary fat pads differ.

In coming months, we will be investigating the effect of including tumor cells in the assay, as well as conditioned media from the tumor cells. The same techniques used to quantitate the number of cells invading the fibrin and the length of the sprouts will be applied to these experiments.

We will also apply these same techniques to similar experiments using blocking antibodies to tPA, uPA, and PAI-1 which will elucidate the role of these proteins in endothelial cell sprouting into fibrin matrices. These experiments will fulfill the aims of the project.

1B. Do growth factors alter DiI-labeled acLDL uptake? In order to quantitate the number of sprouts in each chamber with DiI-labeled acLDL, we would want to know that BPE, FGF, or VEGF treatment did not affect uptake of DiI-labeled acLDL. To ascertain this, we used an immortalized microvascular cell line, HMEC-1. These cells were plated in 75-mm^2 tissue culture flasks, treated with BPE, VEGF, and/or FGF similarly to the explants, and exposed to AlexaFluor 488 - labeled acLDL (Molecular Probes). Cells were harvested and analyzed with flow cytometry. This analysis is depicted in Figure 3. It is seen that fluorescence in the presence or absence of treatments is not substantially different.

1C. Is ε-amino-n-caproic acid (ACA) necessary for fibrin matrix assays or aortic rings or mammary vessels? Others (ref) have added ACA, a plasminogen inhibitor, to fibrin matrix assays at low concentrations to prevent complete digestion of the fibrin due

to activated plasminogen that is the result of tissue damage consequent to dissection of the explant. These investigators have performed analyses of fibrin digestion by use of blocking antibodies to particular plasminogen activators or inhibitors, so it does not seem that this agent interfered with the analyses. However, we were concerned that this agent might be a complicating factor for our assays and wished to see whether we could eliminate it. We performed a concentration-response of ACA starting at 300 μg/ml and going down to 10 μg/ml as well as leaving it out of one assay completely. We did not observe any difference between the chambers that contained various concentrations of ACA or the chambers that contained no ACA (not shown). Therefore, for our assays, it would seem that this agent can be omitted and it will be omitted in future experiments.

2. Prepare and submit manuscript with data from mammary vessel assay. We have experiments done for our first manuscript showing the difference between aortic rings and mammary vessels in fibrin matrices. All that remains is to analyze the data. Work for the second manuscript showing the effect of blocking enzymes for members of the plasminogen activator/inhibitor/receptor family will be completed in the coming year, with preparation of that manuscript following.

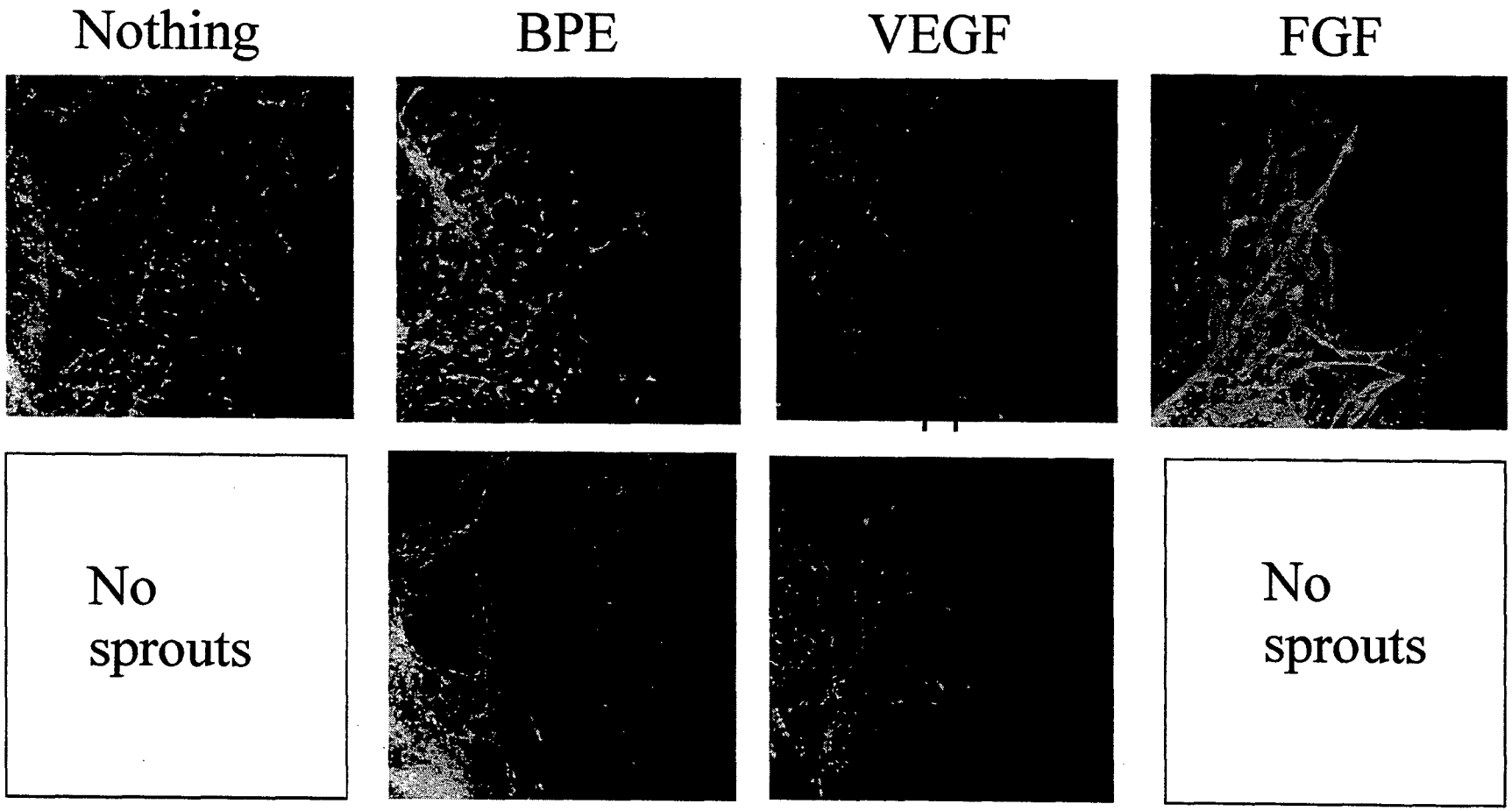

Figure 1A. Mammary vessels (top) and aortic rings (bottom) explants growing in fibrin matrix. Representative fields of 1 of 4 chambers are shown for each treatment. Concentrations of treatments are given in Table 1.Green is Yo-Pro-1 staining. Yellow signifies that green and yellow staining are co-localizing.

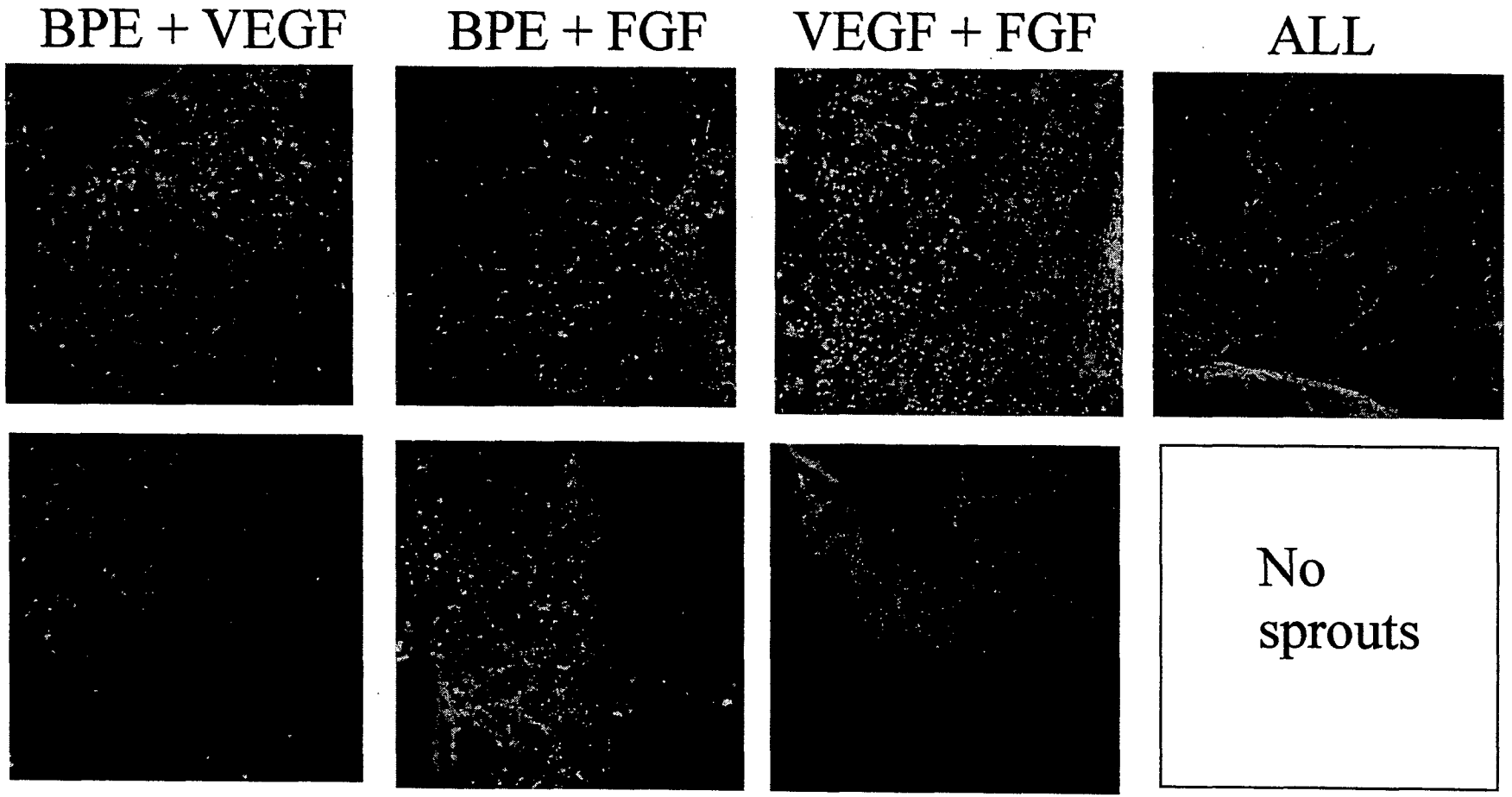

Figure 1B. Mammary vessels (top) and aortic rings (bottom) explants growing in fibrin matrix. Representative chambers of 4 per treatment are shown. Concentrations of treatments are given in Table 1.Green is Yo-Pro-1 staining. Yellow signifies that green and yellow staining are co-localizing.

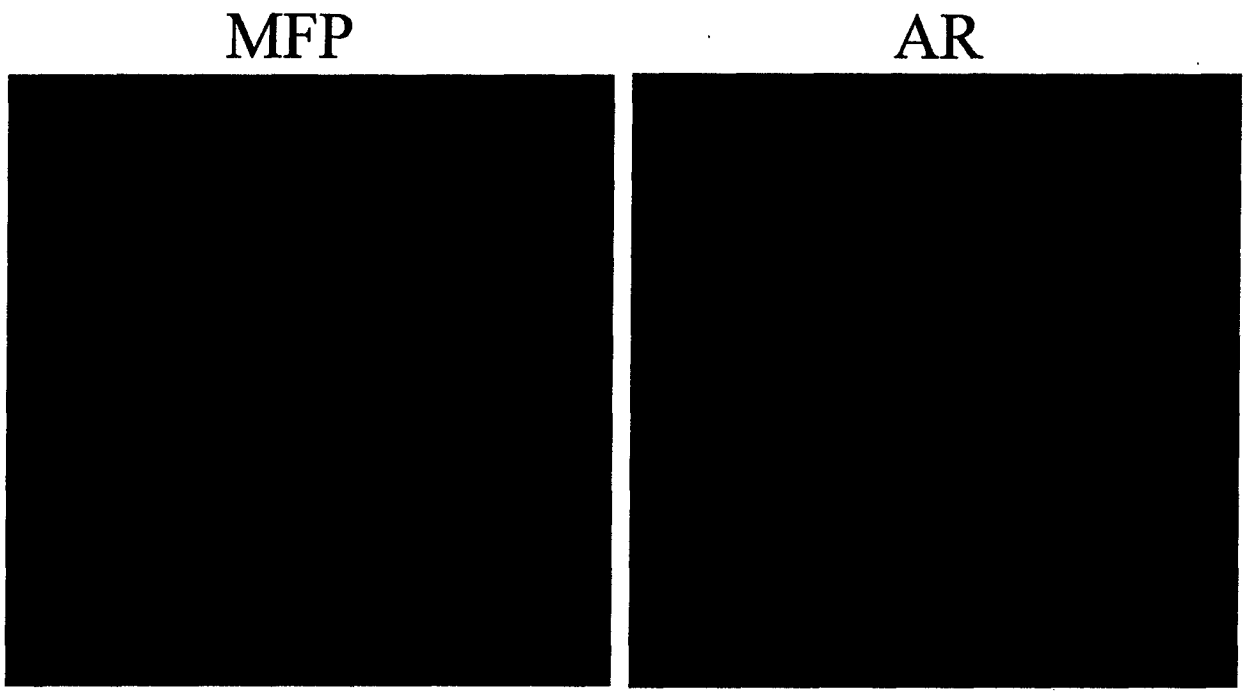

Figure 2. Three dimensional projection of tubular structures sprouting from a mammary vessel (left) or an aortic ring (right) growing in fibrin matrices. Red fluorescence is DiI-labeled Ac-LDL. This view is at approximately 70 degrees from the plane of each section.

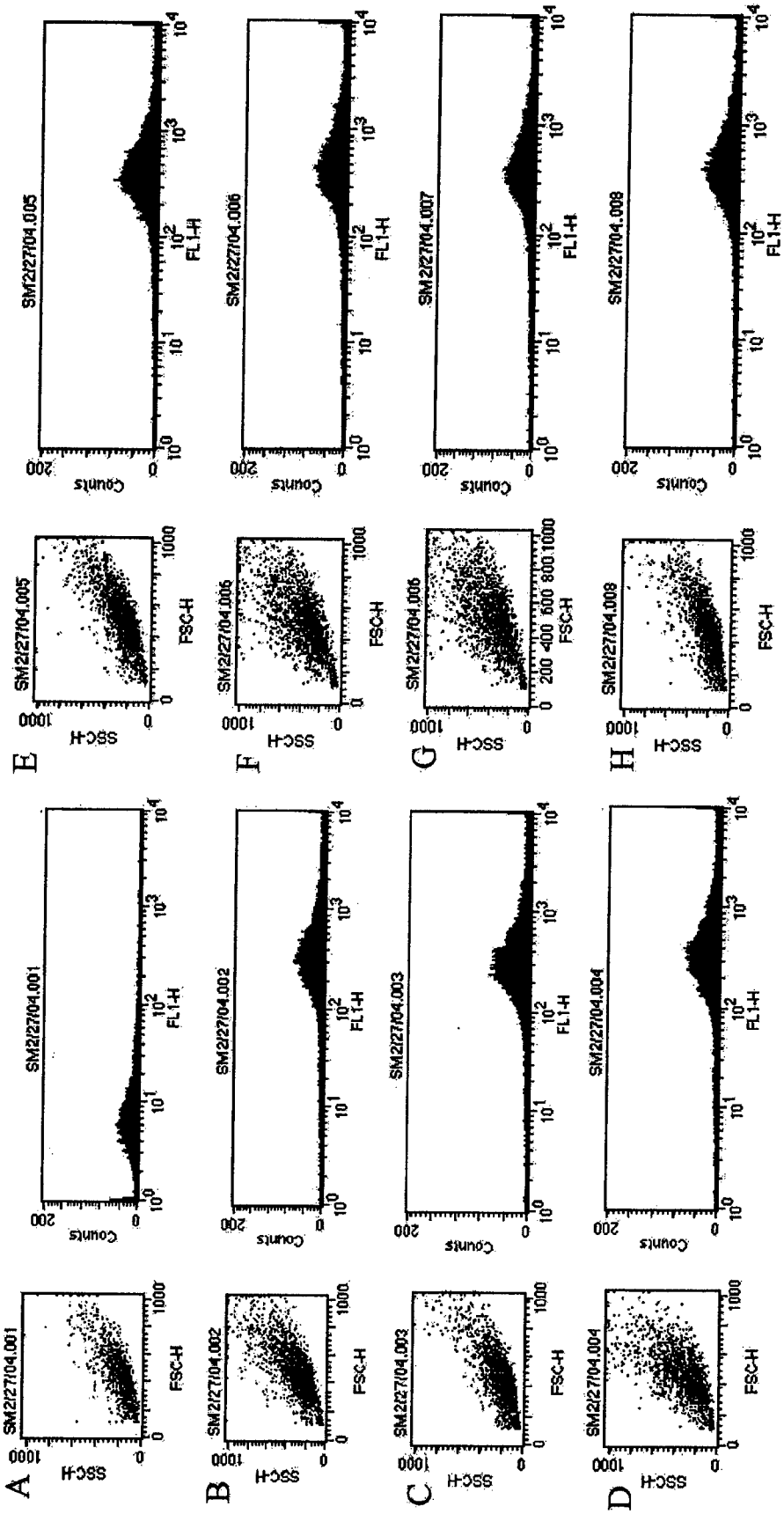

Figure 3. Flow cytometry analysis of HMEC-1 cells treated (B-H) or not treated (A) with AlexaFluor 488 labeled AcLDL. Treatments were as outlined in Table 1. **A** - Basal medium only. **B** - BPE. **C** - VEGF. **D** - FGF. **E** - BPE + VEGF. **F** - BPE + FGF. **G** - VEGF + FGF. **H** - All.

Key Research Accomplishments

1. Completion of a series of experiments comparing mammary vessel explants and aortic ring explants in fibrin gels.

Reportable Outcomes

None.

Conclusions

A no-cost extension was requested to enable us to finish the experiments with our newly-developed in vitro breast cancer angiogenesis model. This model is pertinent to breast cancer angiogenesis since it uses a mouse mammary vessel and a 3-dimensional fibrin matrix. Experiments characterizing this model in contrast with the traditional aortic ring assay have been done and data analysis is in progress. Over the next year, we will complete experiments with the assay incorporating breast cancer cells and/or conditioned media, and blocking antibodies for members of the plasminogen activator/inhibitor/receptor family.

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Appendix

None.